Interspecific Hybridization of Meiotic Parthenogenetic *Meloidogyne chitwoodi* and *M. fallax*

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ABSTRACT

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Hybridization between two meiotic parthenogenetic species of root-knot nematodes, *Meloidogyne chitwoodi* and *M. fallax*, was investigated in two different crossing experiments on tomato plants grown in sand. The first experiment was a controlled cross between the two species. The second experiment was a bulk mating in a 1:1 mixture of two isolates. The haploid chromosome number of the parental isolates was $n = 18$. Successful interspecific hybridization was obtained, and the resulting hybrids produced egg masses. In eggs, cell division was observed, but most of them were without clear differentiation and consequently were sterile. Hatched F_2 juveniles were small in number, not viable, and showed morphological distortions. In the progeny of the isolate mixture of the bulk mating experiment, parental-type females of the two isolates

were present in equal numbers, and 10% of all females were nonviable hybrids. Similar ratios of parental-type and hybrid females were detected in roots of test plants grown in soil from a field sample that contained a mixture of *M. chitwoodi* and *M. fallax* populations. In the controlled cross experiment, isozyme electrophoresis of malate dehydrogenase was applied to distinguish the two species and their hybrids. In the bulk mating experiment, malate dehydrogenase, esterase, and glucose 6-phosphate dehydrogenase were used as markers, two by two simultaneously on the same individual females, providing conclusive evidence for the occurrence of hybrids. This is the first report on interspecific hybridization in *Meloidogyne*. The possible role of interspecific hybridization in species differentiation and interspecific exchange of genetic material within *Meloidogyne* is discussed.

Additional keywords: genetic variation, speciation.

Reproduction in the root-knot nematodes *Meloidogyne chitwoodi* Golden et al. and *M. fallax* Karssen (11,16) is characterized by facultative meiotic parthenogenesis (30), i.e., amphimixis and meiotic parthenogenesis can occur in these species. Amphimixis may result when insemination by males occurs. Until recently, *M. chitwoodi* and *M. fallax* were considered one species, and *M. fallax* has been referred to as *M. chitwoodi* type Baexem (34,37). During the past few years, significant differences between the two species have been reported. Van Meggelen et al. (34) mentioned enzymatic differences between *M. chitwoodi* and *M. chitwoodi* type Baexem and suggested race status. Karssen (17) briefly described morphological and biochemical differences, and Zijlstra et al. (37) showed differences in internal transcribed spacer (ITS) regions of ribosomal DNA. Finally, additional morphological differences between the two species and specific hosts were described (16). These species are distinct from each other in morphological, morphometrical, biochemical, and biological characters, but their reproductive isolation has not been demonstrated.

In a few other nematode species, interspecific hybridization has been proven: *Heterodera* spp. (9,21,26,36), *Globodera* spp. (10, 21,22,28), and *Pratylenchus* spp. (24). The objective of this study was to investigate reproductive isolation in *M. chitwoodi* and *M. fallax*, the results of which might explain the role of interspecific hybridization in genetic divergence in the genus and speciation (19,20). To examine reproductive isolation, hybridization between *M. chitwoodi* and *M. fallax* was studied in a strictly controlled experiment and a bulk mating experiment.

MATERIALS AND METHODS

Nematode history and identification. Crossing experiments were conducted with isolates Ca and Ci of *M. chitwoodi* and isolate Fa of *M. fallax*, all three originated from geographically different sites in the Netherlands (Table 1). *M. javanica* (Treub.) Chitwood, isolate Ja, which originated from China, was used as a reference phenotype in isozyme electrophoresis (Table 1). The isolates were obtained from separated field populations maintained in a greenhouse on tomato (*Lycopersicon esculentum* Mill. 'Moneymaker') and regularly checked for species identity. These isolates were identified as true species by a combination of morphological characteristics (14,16), isozyme patterns (8), and DNA patterns of ITS regions (37) (Table 1).

Controlled crossing experiment. Reciprocal crosses were made between *M. chitwoodi* and *M. fallax*. Isolates Ca and Fa were allowed to hybridize, using a modification of the procedure used by Triantaphyllou (32). Two-week-old tomato seedlings, grown on silversand enriched with 3% slow release fertilizer Osmocote Plus (15 + 11 + 13 [N + P + K] + 2MgO + micros [Scotts Europe BV]) in open-ended transparent plastic tubes (96 ml), were inoculated with prehatched second-stage juveniles (J2). To obtain the paternal line, plants were inoculated on 23 June 1995 with isolates Ca and Fa to a final density of ~45 J2 per ml of soil. This high density enhanced the number of J2 that developed into males, because the gender ratio in *Meloidogyne* is strongly influenced by nematode density (6,29). One week later, roots of additional seedlings were inoculated with nematode suspensions of the same two isolates, at a rate of ~2.5 J2 per ml of soil, to generate the maternal line. This low density caused most of the J2 to develop into females. The plants were placed in a growth cabinet at 20°C, 70% relative humidity (RH), and 16/8 h of light/dark. Fourteen days after inoculation, roots from the paternal line were placed on sieves in a mist cabinet at 100% RH, mist temperature of 28°C, and ambient temperature of 18°C. This high

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humidity caused a thin water film to run from the roots, by which males left the roots and accumulated at the bottom of the receptacle. After 10 days, males were collected and added to the plants containing the maternal line. This procedure was repeated eight times during a period of 17 days, each time adding 10 to 50 males.

Females and their corresponding egg masses were collected separately from roots on 24 August 1995. To examine whether females were inseminated, spermathecae and oocytes of these females were checked for the presence of sperm by making smear preparations (31) that were stained with Hoechst 33258 (procedure used by Albertson et al. [1], with modifications). In inseminated females, male gametes accumulate in the spermatheca, which is a specialized structure in the female gonad. A male gamete fertilizes the oocyte that enters the spermatheca, normally during metaphase I. To detect the required interspecific first generation, hatched J2 of egg masses of inseminated females were inoculated individually on 8 September 1995 on *Solanum tuberosum* L. 'Eigenheimer' root tips grown in petri dishes on water agar, with only one J2 per petri dish. These petri dishes were incubated for 7 weeks under dark conditions in a growth cabinet at 20°C. Females with

their corresponding egg masses were collected separately and used for electrophoresis and a hatching test, respectively.

Bulk mating experiment. A second experiment with isolates Ci and Fa was conducted from June to October 1995 to test the occurrence of spontaneous mating between *M. chitwoodi* and *M. fallax*. The *M. chitwoodi* isolate Ci was different from the isolate used in the controlled cross to investigate whether the possibility of hybridization with different isolates does exist. Two-week-old tomato seedlings were transplanted to 3.2-liter clay pots containing sterilized sandy soil, 33% (vol/vol) water-retaining granules (Hydrocorn; Jongkind Hydro bv, Aalsmeer, the Netherlands), and 3% (wt/vol) Osmocote. One- to two-day-old prehatched J2 were used as inoculum. Three weeks after sowing four tomato seedlings were inoculated, each with ~3.3 J2 per ml of soil, comprising a 1:1 mixture of Ci and Fa juveniles, and placed in a greenhouse at 20°C (18 to 24°C). To complete approximately two nematode generations, the infected plants were maintained for 14 weeks, based on known population development (25). The gender ratio was expected to develop toward a female majority during the first generation, with only a low frequency for possible interspecific hybridization. To detect hybrids and test the viability of the progeny of these hybrids, the experiment was terminated after about the second generation, which approximated the first hybrid generation. From each tomato plant, 40 females with their corresponding egg masses were collected separately and used for electrophoresis and a hatching test, respectively.

Chromosome counting. The chromosomes of the parental lines were examined. Approximately 25 young egg-producing females were collected from infected roots of each parental line, and smear preparations were made (31) and stained with Hoechst 33258. The chromosomes were counted in oocytes during early metaphase I and just after the first maturation division.

Hybrid detection and F₁ progeny viability. Collected females and corresponding egg masses from both experiments were kept at -80 and 20°C, respectively. Due to the facultative parthenogenetic nature of the isolates, females can produce parthenogenetic or sex-

Fig. 1. Malate dehydrogenase zymograms showing representative samples of parental and cross-hybrid phenotypes of individual females of controlled crosses between *Meloidogyne chitwoodi* Ca and *M. fallax* Fa. Lanes 1 and 3, hybrid phenotypes; lanes 4 and 6, maternal *M. chitwoodi* Ca phenotypes; lane 5, absent or null; lanes 9 and 10, parental reference phenotypes for *M. fallax* Fa; lanes 11 and 12, parental reference phenotypes for *M. chitwoodi* Ca. Lanes 2, 7, and 8, *M. javanica* Ja used as a reference phenotype.

TABLE 1. Specific characteristics of the *Meloidogyne* spp. isolates used in this study

Species	Isolate	Sampling year	Original code ^a	<i>EST</i> ^b	<i>MDH</i> ^c	<i>Hinf</i> I ^d	<i>Rsa</i> I ^e
<i>M. chitwoodi</i>	Ca	1991	C3022 (PD)	S1	N1a	440, 270	760
<i>M. chitwoodi</i>	Ci	1991	C5273-C (PD)	S1	N1a	440, 270	760
<i>M. fallax</i>	Fa	1992	CHB (PAGV)	F3	N1b	430, 270	620
<i>M. javanica</i>	Ja	1990	C3059 (PD)	J3	N1	440, 320	760

^a PD = Plant Protection Service, Wageningen, the Netherlands. PAGV = Research Station for Arable Farming and Field Production of Vegetables, Lelystad, the Netherlands.

^b Isozyme phenotype for esterase (6,15). F3 = Unique *EST* phenotype for *M. fallax*.

^c Isozyme phenotype for malate dehydrogenase (6). N1b = Unique *MDH* phenotype for *M. fallax*.

^d Discriminating restriction products (in base pairs) when restricted with *Hinf*I of the 760-bp polymerase chain reaction-amplified internal transcribed spacer (ITS) regions (33).

^e Discriminating restriction product (in base pairs) when restricted with *Rsa*I of the 760-bp polymerase chain reaction-amplified ITS regions (33).

Fig. 2. Eggs of *Meloidogyne chitwoodi* × *M. fallax* hybrids at ~1 month after hatching, stained with Hoechst 33258. **A**, Undifferentiated mass of nuclei. **B**, Malformed juvenile.

ual offspring. The latter may be the result of insemination of females by either males of the maternal or paternal line. Moreover, more than one male may fertilize one female. Consequently, parthenogenetically and sexually produced eggs may occur in an egg mass of an inseminated female, and the progeny of a crossing may contain both parthenogenetic and sexual offspring. In such a progeny, true hybrid females can be distinguished from a cross with a male from the maternal line and a parthenogenetic offspring by a differentiating marker between the two species. Three independent isozyme markers were used: malate dehydrogenase (*MDH*; EC 1.1.1.37), esterase (*EST*; EC 3.1.1.1), and glucose 6-phosphate dehydrogenase (*G6PD*; EC number 1.1.1.49). *MDH* was applied in the controlled crossing experiment, and *MDH*, *EST*, and *G6PD* were applied in the bulk mating experiment. To provide conclusive evidence for hybridization, individual females were subjected to isozyme electrophoreses of *MDH* and *EST*, and other females were subjected to electrophoresis of *MDH* and *G6PD*, resulting in two phenotypes for one female with independent isozyme markers. Isozyme electrophoresis and staining were carried out on microgels according to Karszen et al. (18). Egg masses were collected individually in microvials with 10 ml of water and placed in a 20°C incubator to hatch. During a period of 8 weeks, hatching was checked weekly.

RESULTS

Hybrids in the controlled crossing experiment. The haploid chromosome number of both parental isolates in the controlled crossing experiment, Ca and Fa, was the same ($n = 18$). No F_1

Fig. 3. Characteristic head and tail structures of a juvenile of a *Meloidogyne chitwoodi* × *M. fallax* hybrid compared with its parents. **A and D**, *M. chitwoodi*. **B and E**, Progeny of hybrid. **C and F**, *M. fallax*.

hybrids were obtained from the cross of *M. fallax* and *M. chitwoodi*, with *M. fallax* as the female parent, because the *M. chitwoodi* male parent did not produce sufficient males for the experiment. This has been noticed often during maintenance of *M. chitwoodi* isolates. F₁ hybrids were obtained from the cross of *M. chitwoodi* and *M. fallax*, with *M. chitwoodi* as the female parent. Seven females were collected from two plants, of which one female contained sperm, which was detected in the spermatheca and oocytes. Originating from the egg mass of this inseminated female, 60 hatched J2 were inoculated separately on root tips, resulting in 21 females with egg masses. Electrophoresis for *MDH* resulted in two isozyme patterns: eight females exhibited only the maternal (*M. chitwoodi*) isozyme band (Fig. 1, lanes 4 and 6), and four females exhibited patterns, with three regularly spaced bands corresponding to the two parental bands and an intermediate band (Fig. 1, lanes 1 and 3). For nine females, no clear bands were found (Fig. 1, lane 5), probably due to loss of female material during preparation of the electrophoresis samples. Although a successful cross was obtained, none of the four hybrid females with clear intermediate bands produced normal fertile offspring, in contrast to all of the females showing maternal isozyme patterns. Of the nine females without clear isozyme bands, two produced offspring, and seven did not. Assuming that these 7 were also true hybrids, a total of 11 interspecific hybrid females and 10 parthenogenetic progeny was obtained. Egg masses of hybrid females contained up to 100 eggs each. Most eggs were able to develop into a multicellular undifferentiated structure, and in some eggs, a juvenile was visible (Fig. 2). Some juveniles were even able to hatch at 20°C but were distorted, showing deviating head and tail shapes (Fig. 3), and died shortly after hatching.

Hybrids in the bulk mating experiment. The haploid chromosome number of isolate Ci, used as one parent in the bulk mating experiment of *M. chitwoodi* with *M. fallax*, was $n = 18$, like the isolates Ca and Fa. As a result of this crossing experiment, 77 and 67 parental-type females of *M. chitwoodi* and *M. fallax*, respectively, were obtained and detected, with *MDH* as marker, 14 weeks after inoculation (Table 2; Fig. 4). Additionally, 16 F₁ hybrid females were detected, which was 10% of the total number of examined females. None of the hybrid females produced viable offspring, whereas all parental-type females produced normal progeny. Parental-type and hybrid females showed corresponding parental and hybrid patterns for the two isozymes *MDH* and *EST* (Fig. 5), as well as for *MDH* and *G6PD* (Fig. 6) on the zymograms, comparable to *MDH* (Fig. 1) in the controlled crossing experiment.

DISCUSSION

This is the first report of successful interspecific crossing within the genus *Meloidogyne*, demonstrating hybridization between *M. chitwoodi* and *M. fallax*. Ample evidence for interspecific hybridization was obtained in this study from controlled and bulk crosses. Triantaphyllou successfully hybridized *M. hapla* isolates ($n = 15$ [♀] and 17 [♂] chromosomes) (A. C. Triantaphyllou, *personal communication*). Hybrid progeny had $n = 16$ or 17 chro-

mosomes. Pairing of homologous chromosomes was not perfect, because loops were observed in some bivalents during diakinesis; nevertheless, hybrid progeny were propagated successfully for many generations. Attempts to hybridize *M. chitwoodi* with *M. hapla*, however, were unsuccessful, as detected with chromosome and biochemical markers (A. C. Triantaphyllou, *personal communication*; 33). In this study, the isolates used for crosses appeared to possess identical chromosome numbers. No data are available on crosses between *M. chitwoodi* and *M. fallax* with different chromosome numbers. Differences in chromosome number between parental isolates are expected to interfere with hybridization.

The current study supports the separate species status of *M. chitwoodi* and *M. fallax*, based on the nonviable progenies of their interspecific hybrids. Triantaphyllou (30) stated that "...conceptually they (facultatively meiotic parthenogenetic species) can be considered as true biological species if they are really reproductively isolated from each other. However, tests for demonstrating reproductive isolation are technically very difficult because of the facultative nature of parthenogenetic reproduction and the lack of genetic markers that would verify intercrossing." By demonstrating reproductive isolation between the two meiotic parthenogenetic species in this study, it is now evident that they are true biological species.

Isozyme electrophoresis was a convenient tool for detecting interspecific hybridization, using isozymes as genetic markers, as

Fig. 4. Malate dehydrogenase zymograms showing representative samples of parental and cross-hybrid phenotypes of the bulk mating experiment between *Meloidogyne chitwoodi* (Ci) and *M. fallax* (Fa) and phenotypes of mixtures of females of the two species. Lanes 2, 4, 10, 18, 20, 21, 23, 28, and 29, phenotypes of *M. chitwoodi*. Lanes 1, 3, 7, 8, 11, 12, 13, 17, 19, 27, and 30, phenotypes of *M. fallax*. Lanes 9, 16 and 26, hybrids. Lanes 22, 24, and 25, species mixtures. Lanes 5, 6, 14, and 15, *M. javanica* (Ja) used as a reference phenotype.

TABLE 2. Number of parental-type and hybrid females in the bulk mating experiment of *Meloidogyne chitwoodi* isolate Ci and *M. fallax* isolate Fa

Tomato plant	Ci ^a	Fa ^b	Hybrid ^c	Total ♀♀
1	16	20	4	40
2	24	12	4	40
3	17	18	5	40
4	20	17	3	40
Total	77	67	16	160

^a Parental-type females possessing the N1a band (*MDH* phenotype of Ci).

^b Parental-type females possessing the N1b band (*MDH* phenotype of Fa).

^c Hybrid-type females possessing the N1a and N1b bands and an intermediate band.

has been shown previously in certain insects, for example water-flea (*Daphnia* spp.) (35), stick-insects (*Bacillus* spp.) (3), and planthopper (*Muellerianella* spp.) (2). Van Meggelen et al. (34) stated that *M. chitwoodi* and *M. fallax* have different *MDH* phenotypes. The hybrid was expected to possess three distinct bands in a zymogram: both parental bands and an intermediate band, because *MDH* consists of two polypeptide chains, and a heterodimer is expected to be formed in a heterozygote (23), in contrast to a mixture of species that show only the two parental bands (Fig. 4). The three *MDH* patterns found in this study correspond to genotypes for the two parental species and their hybrid, confirming the codominant nature of locus *MDH*. *MDH* is a stable marker for distinguishing species of *Meloidogyne*, showing identical isozyme patterns within one species, with a few exceptions in *M. arenaria*, *M. incognita*, and *M. javanica* (7). *MDH* genotypes of ~100 females of *M. chitwoodi* and 60 of *M. fallax*, representing various isolates, were characteristic for species, showing identical phenotypes, as described in this paper (J. G. van der Beek and G. Karssen, unpublished data). The genotypes of the hybrid females in this study, which exhibit hybrid patterns for the independent markers *MDH* and *EST* and for *MDH* and *G6PD* in the same individuals, provide conclusive evidence for hybridization between *M. chitwoodi* and *M. fallax* (Figs. 5 and 6). *G6PD*, which distinguishes *M. chitwoodi* from *M. fallax* (4), is also a heterodimer, and in the hybrid, the middle band

is predominantly visible. *EST* is a monomer, and its phenotype for *M. fallax* has been described as a "null" (16). However, when stained for 90 min, a faint three-band pattern appears that has not been described yet and is denoted as F3 (Fig. 5, lanes 2 and 5). The hybrid *EST* pattern shows a strong band corresponding to the *M. chitwoodi* S1 phenotype and the slower band of the *M. fallax* F3 phenotype (Fig. 5). The *MDH* phenotype of *M. fallax*, although described previously (16), is designated as N1b.

Not all females were expected to be fertilized by males. Mating in parthenogenetic *Meloidogyne* spp. occurs by chance (27). However, even if females were inseminated, it could have been entirely or partly by males of the maternal isolate. Additionally, the egg mass from an inseminated female was expected to contain an unknown portion of unfertilized, parthenogenetic eggs. This explains the occurrence of maternal-type females in the progeny of an inseminated female in the controlled crossing experiment. Furthermore, it explains why a Mendelian segregation is not expected in crossings between meiotic parthenogenetic *Meloidogyne* spp.

Interestingly, the two species used in this study are either allopatric or sympatric; both are prevalent in the southeastern Netherlands. Because of their similarity in ecological niche, in total protein patterns (J. G. van der Beek, unpublished data), and in sharing many host plants, including monocotyledons, it could be that both species share a common ancestry. In our experiments

Fig. 5. Zymograms of malate dehydrogenase (*MDH*) and esterase (*EST*), showing representative samples of parental and cross-hybrid phenotypes for both isozymes on the same females used in the bulk mating between *Meloidogyne chitwoodi* (Ci) and *M. fallax* (Fa). Lane 4, phenotypes of *M. chitwoodi*. Lanes 1, 2, and 5, phenotypes of *M. fallax*. Lane 3, phenotypes of the hybrid. Lane 6, *M. javanica* (Ja) used as a reference. *MDH* and *EST* phenotypes for *M. fallax* are designated as N1b and F3, respectively.

Fig. 6. Zymograms of malate dehydrogenase (*MDH*) and glucose 6-phosphate dehydrogenase (*G6PD*) showing representative samples of parental and cross-hybrid phenotypes for both isozymes on the same females used in the bulk mating between *Meloidogyne chitwoodi* (Ci) and *M. fallax* (Fa). Lane 3, phenotypes of *M. chitwoodi*. Lanes 2, 4, and 6, phenotypes of *M. fallax*. Lane 5, phenotypes of the hybrid. Lane 1, *M. javanica* (Ja) used as a reference.

with mixtures of isolates, hybrids between *M. chitwoodi* and *M. fallax* occurred regularly. Although deformed and nonviable, a very few juveniles were found in the offspring of these hybrids. Speculatively, hybrids may occasionally produce fertile progeny, because they are able to backcross to one of the parents, as is shown in interspecific hybrids in *Daphnia* (12,35). For *Meloidogyne*, this would signify that exchange of DNA could be possible, including genes for avirulence/virulence. If so, successful breeding for durable crop plant resistance should include testing of isolates of all potentially aggressive species that can interbreed.

In addition to broadening the genetic base of a species by backcrosses of the hybrid to a parental population, hybridization also may play an important role in speciation in certain organisms (19). Two steps are required for a species to arise from interspecific hybridization (3): interspecific hybridization and changes in the maturation divisions that allow transmission of the hybrid genome to the next generation. The latter changes are undoubtedly rare. Dalmasso and Bergé (5) speculated that *M. javanica*, a polyploid, mitotic, parthenogenetically reproducing species, may have arisen in this way, because of its high level of heterozygosity for some isozymes. One of its parents could have been *M. hapla*, based on similarity in nucleotide sequences between *M. javanica* and *M. hapla* (13).

In addition to the results described in this paper, more evidence for hybridization between *M. chitwoodi* and *M. fallax* was derived from observations on lettuce roots in which parental and hybrid females were detected. Four of forty females showed hybrid *MDH* phenotypes similar to those presented in Figures 1 and 4. These lettuce plants were grown in soil that originated from a field in which an almost equal mixture of populations of the two species was detected.

Mixtures of *Meloidogyne* species are found more frequently under natural conditions. In these situations, hybridization may be feasible, and under environmentally favorable conditions for host plant rotation, intensity of land use, and soil characteristics, it is likely that new forms of *Meloidogyne* may appear.

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